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(54) Title: LACCASE MUTANTS

(57) Abstract

The present invention relates to laccase mutants with increased oxidation potential and/or changed pH optimum and/or altered mediator pathway and/or altered O_2/OH^-pathway.

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LACCASE MUTANTS

FIELD OF THE INVENTION

The present invention relates to laccase mutants with increased oxidation potential and/or changed pH optimum and/or altered mediator pathway and/or altered O_2/OH^-pathway.

BACKGROUND OF THE INVENTION

Laccase is a polyphenol oxidase (EC 1.10.3.2) which catalyses the oxidation of a variety of inorganic and aromatic compounds, particularly phenols, with the concomitant reduction of molecular oxygen to water.

Laccase belongs to a family of blue copper-containing oxidases which includes ascorbate oxidase and the mammalian plasma protein ceruloplasmin. All these enzymes are multi-copper-containing proteins.

Because laccases are able to catalyze the oxidation of a variety of inorganic and aromatic compounds, laccases have been suggested in many potential industrial applications such as lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, hair colouring, and waste water treatment.

The various applications ask for laccases with specific properties. It is the purpose of the present application to create laccase variants with increased oxidation potential and/or changed pH optimum and/or altered mediator pathway and/or altered O_2/OH^--pathway.

BRIEF DISCLOSURE OF THE INVENTION

The present invention relates to laccase variants, in particular to

- A variant of a parent laccase, which variant has laccase activity, and increased oxidation potential and comprises a mutation in a position corresponding to at least one of the following positions:

G511A,V,P,L,I,F,Y,W;
T428A,V,P,L,I,F,Y,W;
S510A,V,P,L,I,F,Y,W;
D106A,V,P,L,I,F,Y,W;
N109A,V,P,L,I,F,Y,W,Q;
L500I,F,Y,W;
A108V,P,L,I,F,Y,W;
G514A,V,P,L,I,F,Y,W;
wherein the parent laccase has the amino acid sequence given in SEQ ID No. 1 or the parent laccase has an amino acid sequence which is at least 80% homologous to SEQ ID No. 1;

- A variant of a parent laccase, which variant has laccase activity and an altered pH optimum and comprises a mutation in a position corresponding to at least one of the following positions:
192-193;
234-236;
269;
293-294;
364-365;
372-373;
426-433;
503-513;
wherein the parent laccase has the amino acid sequence given in SEQ ID No. 1 or the parent laccase has an amino acid sequence which is at least 80% homologous to SEQ ID No. 1;

- A variant of a parent laccase, which variant has laccase activity and an altered mediator efficiency and comprises a mutation in a position corresponding to at least one of the following positions:
185-194;
235;
293-294;
365-373;
427-429;

505;
507-508;
510-511;
wherein the parent laccase has the amino acid sequence given in SEQ ID No. 1 or the parent laccase has an amino acid sequence which is at least 80% homologous to SEQ ID No. 1; and

- A variant of a parent laccase, which variant has laccase activity and an altered O_2/OH^--pathway and comprises a mutation in a position corresponding to at least one of the following positions:
A506E;
N109D;
H93E;
H95E;
M433E;
M480E;
wherein the parent laccase has the amino acid sequence given in SEQ ID No. 1 or the parent laccase has an amino acid sequence which is at least 80% homologous to SEQ ID No. 1.

In still further aspects the invention relates to DNA encoding such variants and methods of preparing the variants.

Finally, the invention relates to the use of the variants for various industrial purposes.

DETAILED DISCLOSURE OF THE INVENTION

Homologous Laccases

A number of laccases produced by different fungi are homologous on the amino acid level. For instance, when using the homology percent obtained from UWGCG program using the GAP program with the default parameters (penalties: gap weight=3.0, length weight=0.1; WISCONSIN PACKAGE Version 8.1-UNIX, August 1995, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) the following homology was found:

Myceliophthora thermophila laccase comprising the amino acid sequence shown in SEQ ID No. 1: 100%;
Scytalidium thermophilum laccase comprising the amino acid sequence shown in SEQ ID No. 2: 81.2%.

Because of the homology found between the above mentioned laccases, they are considered to belong to the same class of laccases, namely the class of "*Myceliophthora*-like laccases".

Accordingly, in the present context, the term "*Myceliophthora*-like laccase" is intended to indicate a laccase which, on the amino acid level, displays a homology of at least 80% to the *Myceliophthora* laccase SEQ ID NO 1, or a laccase which, on the amino acid level, displays a homology of at least 85% to the *Myceliophthora* laccase SEQ ID NO 1, or a laccase which, on the amino acid level, displays a homology of at least 90% to the *Myceliophthora* laccase SEQ ID NO 1, or a laccase which, on the amino acid level, displays a homology of at least 95% to the *Myceliophthora* laccase SEQ ID NO 1, or a laccase which, on the amino acid level, displays a homology of at least 98% to the *Myceliophthora* laccase SEQ ID NO 1.

In the present context, "derived from" is intended not only to indicate a laccase produced or producible by a strain of the organism in question, but also a laccase encoded by a DNA sequence isolated from such strain and produced in a host organism containing said DNA sequence. Finally, the term is intended to indicate a laccase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the laccase in question.

Variants with altered oxidation potential

The redox potentials of various wild type laccases have been found to be the following (measured at pH 5.3):

	E°, V *vs* NHE
Myceliophthora thermophila (SEQ ID No. 1):	0.48
Scytalidium thermophilum (SEQ ID No. 2):	0.53

It is contemplated that it is possible to increase the oxidation potential of a parent laccase, wherein said variant is the result of a mutation, i.e. one or more amino acid residues have been deleted from, replaced or added to the parent laccase. Preferred positions for mutations are the following:

Myceliophthora thermophila laccase (SEQ ID No. 1):
G511A,V,P,L,I,F,Y,W;
T428A,V,P,L,I,F,Y,W;
S510A,V,P,L,I,F,Y,W;
D106A,V,P,L,I,F,Y,W;
N109A,V,P,L,I,F,Y,W,Q;
L500I,F,Y,W;
A108V,P,L,I,F,Y,W;
G514A,V,P,L,I,F,Y,W; in particular
G511A,V,L,I,F;
T428V;
S510V;
D106L;
N109I,F,Q;
L500F;
A108V,I;
G514A,V,L,I,F.

Preferred variants include any combination of the above mentioned mutations.

Variants with altered pH optimum

The desired pH optimum of a laccase depends on which application is of interest, e.g., if the laccase is to be used for denim bleaching the preferred pH optimum will be around pH 5-8, whereas if the laccase is to be used for washing purposes the preferred pH optimum will be around pH 8-10.

It is contemplated that it is possible to alter the pH optimum of a parent laccase wherein said variant is the result of a mutation, i.e. one or more amino acid residues have been deleted from, replaced or added to the parent laccase. Preferred

positions for mutations are the following:

Myceliophthora thermophila (SEQ ID No. 1):
192-193;
234-236;
269;
293-294;
364-365;
372-373;
426-433;
503-513.

Preferred substitutions are the following: E, D, L, I, F, Y, W.

Variants with altered mediator efficiency

Laccases are often used in combination with so called mediators or enhancers, e.g., in combination with phenothiazine or phenothiazine related compounds (see WO 95/01426) or in combination with acetosyringone or acetosyringone related compounds (see WO 96/10079).

It is contemplated that it is possible to alter the mediator efficiency (in order to make the mediator more efficient), of a parent laccase wherein said variant is the result of a mutation, i.e. one or more amino acid residues have been deleted from, replaced or added to the parent laccase. Preferred positions for mutations are the following:

Myceliophthora thermophila laccase (SEQ ID No. 1):
185-194;
235;
293-294;
365-373;
427-429;
505;
507-508;
510-511.

Preferred substitutions are in particular one or more of the

following mutations:
N189G,A,S,T;
S190G,A;
F371* (deletion);
F371G,A.

Variants with altered O_2/OH^--pathway

It is contemplated that it is possible to lower the possibility of OH^- entering the trinuclear Cu site by producing one or more of the following mutations:

Myceliophthora thermophila (SEQ ID No. 1):
A506E;
N109D;
H93E;
H95E;
M433E;
M480E.

Methods of preparing laccase variants

Several methods for introducing mutations into genes are known in the art. After a brief discussion of the cloning of laccase-encoding DNA sequences, methods for generating mutations at specific sites within the laccase-encoding sequence will be discussed.

Cloning a DNA sequence encoding a laccase

The DNA sequence encoding a parent laccase may be isolated from any cell or microorganism producing the laccase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the laccase to be studied. Then, if the amino acid sequence of the laccase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify laccase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known laccase gene could be used as a probe to identify laccase-encoding clones, using hybridization and washing conditions of lower stringency.

A method for identifying laccase-encoding clones involves inserting cDNA into an expression vector, such as a plasmid, transforming laccase-negative fungi with the resulting cDNA library, and then plating the transformed fungi onto agar containing a substrate for laccase, thereby allowing clones expressing the laccase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method. In the phosphoroamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers.

Site-directed mutagenesis

Once a laccase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the laccase-encoding sequence, is created in a vector carrying the laccase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with T7 DNA polymerase and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into laccase-encoding DNA sequences is described in Nelson and Long (1989). It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random mutagenesis

The random mutagenesis of a DNA sequence encoding a parent laccase may conveniently be performed by use of any method known in the art.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing

agents.

The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the laccase enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase and ligase.

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent laccase enzyme is subjected to PCR under conditions that increase the misincorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cereviseae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the laccase enzyme by e.g. transforming a plasmid containing the parent enzyme into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may subsequently be

transformed into the expression organism.

The DNA sequence to be mutagenized may conveniently be present in a genomic or cDNA library prepared from an organism expressing the parent laccase enzyme. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenizing agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to the expression step or the screening step being performed. Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenizing agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are fungal hosts such as *Aspergillus niger* or *Aspergillus oryzae*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

Localized random mutagenesis

The random mutagenesis may advantageously be localized to a part of the parent laccase in question. This may, e.g., be

advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized random mutagenesis is conveniently performed by use of PCR-generated mutagenesis techniques as described above or any other suitable technique known in the art.

Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g. by being inserted into a suitable vector, and said part may subsequently be subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

With respect to the screening step in the above-mentioned method of the invention, this may conveniently be performed by use of aa filter assay based on the following principle:

A microorganism capable of expressing the mutated laccase enzyme of interest is incubated on a suitable medium and under suitable conditions for the enzyme to be secreted, the medium being provided with a double filter comprising a first protein-binding filter and on top of that a second filter exhibiting a low protein binding capability. The microorganism is located on the second filter. Subsequent to the incubation, the first filter comprising enzymes secreted from the microorganisms is separated from the second filter comprising the microorganisms. The first filter is subjected to screening for the desired enzymatic activity and the corresponding microbial colonies present on the second filter are identified.

The filter used for binding the enzymatic activity may be any protein binding filter e.g. nylon or nitrocellulose. The top filter carrying the colonies of the expression organism may be any filter that has no or low affinity for binding proteins e.g. cellulose acetate or Durapore™. The filter may be pretreated with any of the conditions to be used for screening or may be treated during the detection of enzymatic activity.

The enzymatic activity may be detected by a dye,

fluorescence, precipitation, pH indicator, IR-absorbance or any other known technique for detection of enzymatic activity.

The detecting compound may be immobilized by any immobilizing agent, e.g., agarose, agar, gelatine, polyacrylamide, starch, filter paper, cloth; or any combination of immobilizing agents.

Laccase activity

In the context of this invention, the laccase activity was measured using 10-(2-hydroxyethyl)-phenoxazine (HEPO) as substrate for the various laccases. HEPO was synthesized using the same procedure as described for 10-(2-hydroxyethyl)-phenothiazine, (G. Cauquil in Bulletin de la Society Chemique de France, 1960, p. 1049). In the presence of oxygen laccases (E.C. 1.10.3.2) oxidize HEPO to a HEPO radical that can be monitored photometrically at 528 nm.

The *Myceliophthora thermophila* laccase was measured using 0.4 mM HEPO in 25 mM Tris-HCl, pH 7.5, 0.05% TWEEN-20 at 30 °C. The absorbance at 528 nm was followed for 200 s and the rate calculated from the linear part of the progress curve.

Expression of laccase variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding a laccase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a

bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a laccase variant of the invention, especially in a fungal host, are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the laccase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene, the product of which complements a defect in the host cell, such as one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

The procedures used to ligate the DNA construct of the inven-

tion encoding a laccase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. (1989)).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a laccase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a fungal cell.

The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method of producing a laccase variant of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the laccase variant of the invention.

Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The laccase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Industrial Applications

The laccase variants of this invention possesses valuable properties allowing for various industrial applications, in particular lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, hair dyeing, textile dyeing, bleaching of textiles (in particular bleaching of denim as described in WO 96/12845 and WO 96/12846) and waste water treatment.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(ii) TITLE OF INVENTION: LACCASE MUTANTS

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 573 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Gln Gln Ser Cys Asn Thr Pro Ser Asn Arg Ala Cys Trp Thr Asp Gly
1 5 10 15

Tyr Asp Ile Asn Thr Asp Tyr Glu Val Asp Ser Pro Asp Thr Gly Val

20 25 30

Val Arg Pro Tyr Thr Leu Thr Leu Thr Glu Val Asp Asn Trp Thr Gly
35 40 45

Pro Asp Gly Val Val Lys Glu Lys Val Met Leu Val Asn Asn Ser Ile
50 55 60

Ile Gly Pro Thr Ile Phe Ala Asp Trp Gly Asp Thr Ile Gln Val Thr
65 70 75 80

Val Ile Asn Asn Leu Glu Thr Asn Gly Thr Ser Ile His Trp His Gly
85 90 95

Leu His Gln Lys Gly Thr Asn Leu His Asp Gly Ala Asn Gly Ile Thr
100 105 110

Glu Cys Pro Ile Pro Pro Lys Gly Gly Arg Lys Val Tyr Arg Phe Lys
115 120 125

Ala Gln Gln Tyr Gly Thr Ser Trp Tyr His Ser His Phe Ser Ala Gln
130 135 140

Tyr Gly Asn Gly Val Val Gly Ala Ile Gln Ile Asn Gly Pro Ala Ser
145 150 155 160

Leu Pro Tyr Asp Thr Asp Leu Gly Val Phe Pro Ile Ser Asp Tyr Tyr
165 170 175

Tyr Ser Ser Ala Asp Glu Leu Val Glu Leu Thr Lys Asn Ser Gly Ala
180 185 190

Pro Phe Ser Asp Asn Val Leu Phe Asn Gly Thr Ala Lys His Pro Glu
195 200 205

Thr Gly Glu Gly Glu Tyr Ala Asn Val Thr Leu Thr Pro Gly Arg Arg
210 215 220

His Arg Leu Arg Leu Ile Asn Thr Ser Val Glu Asn His Phe Gln Val
225 230 235 240

Ser Leu Val Asn His Thr Met Cys Ile Ile Ala Ala Asp Met Val Pro
245 250 255

Val Asn Ala Met Thr Val Asp Ser Leu Phe Leu Gly Val Gly Gln Arg
260 265 270

Tyr Asp Val Val Ile Glu Ala Asn Arg Thr Pro Gly Asn Tyr Trp Phe
275 280 285

Asn Val Thr Phe Gly Gly Gly Leu Leu Cys Gly Gly Ser Arg Asn Pro
290 295 300

Tyr Pro Ala Ala Ile Phe His Tyr Ala Gly Ala Pro Gly Gly Pro Pro
305 310 315 320

Thr Asp Glu Gly Lys Ala Pro Val Asp His Asn Cys Leu Asp Leu Pro
325 330 335

Asn Leu Lys Pro Val Val Ala Arg Asp Val Pro Leu Ser Gly Phe Ala
340 345 350

Lys Arg Ala Asp Asn Thr Leu Asp Val Thr Leu Asp Thr Thr Gly Thr
355 360 365

Pro Leu Phe Val Trp Lys Val Asn Gly Ser Ala Ile Asn Ile Asp Trp
370 375 380

Gly Arg Ala Val Val Asp Tyr Val Leu Thr Gln Asn Thr Ser Phe Pro
385 390 395 400

Pro Gly Tyr Asn Ile Val Glu Val Asn Gly Ala Asp Gln Trp Ser Tyr
405 410 415

Trp Leu Ile Glu Asn Asp Pro Gly Ala Pro Phe Thr Leu Pro His Pro
420 425 430

Met His Leu His Gly His Asp Phe Tyr Val Leu Gly Arg Ser Pro Asp
435 440 445

Glu Ser Pro Ala Ser Asn Glu Arg His Val Phe Asp Pro Ala Arg Asp
450 455 460

Ala Gly Leu Leu Ser Gly Ala Asn Pro Val Arg Arg Asp Val Ser Met
465 470 475 480

Leu Pro Ala Phe Gly Trp Val Val Leu Ser Phe Arg Ala Asp Asn Pro
485 490 495

Gly Ala Trp Leu Phe His Cys His Ile Ala Trp His Val Ser Gly Gly
500 505 510

Leu Gly Val Val Tyr Leu Glu Arg Ala Asp Asp Leu Arg Gly Ala Val
515 520 525

Ser Asp Ala Asp Ala Asp Asp Leu Asp Arg Leu Cys Ala Asp Trp Arg
530 535 540

Arg Tyr Trp Pro Thr Asn Pro Tyr Pro Lys Ser Asp Ser Gly Leu Lys
545 550 555 560

His Arg Trp Val Glu Glu Gly Glu Trp Leu Val Lys Ala
565 570

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 616 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Lys Arg Phe Phe Ile Asn Ser Leu Leu Leu Leu Ala Gly Leu Leu
1 5 10 15

Asn Ser Gly Ala Leu Ala Ala Pro Ser Thr His Pro Arg Ser Asn Pro
20 25 30

Asp Ile Leu Leu Glu Arg Asp Asp His Ser Leu Thr Ser Arg Gln Gly
35 40 45

Ser Cys His Ser Pro Ser Asn Arg Ala Cys Trp Cys Ser Gly Phe Asp
50 55 60

Ile Asn Thr Asp Tyr Glu Thr Lys Thr Pro Asn Thr Gly Val Val Arg

65 70 75 80

Arg Tyr Thr Phe Asp Ile Thr Glu Val Asp Asn Arg Pro Gly Pro Asp
85 90 95

Gly Val Ile Lys Glu Lys Leu Met Leu Ile Asn Asp Lys Leu Leu Gly
100 105 110

Pro Thr Val Phe Ala Asn Trp Gly Asp Thr Ile Glu Val Thr Val Asn
115 120 125

Asn His Leu Arg Thr Asn Gly Thr Ser Ile His Trp His Gly Leu His
130 135 140

Gln Lys Gly Thr Asn Tyr His Asp Gly Ala Asn Gly Val Thr Glu Cys
145 150 155 160

Pro Ile Pro Pro Gly Gly Ser Arg Val Tyr Ser Phe Arg Ala Arg Gln
165 170 175

Tyr Gly Thr Ser Trp Tyr His Ser His Phe Ser Ala Gln Tyr Gly Asn
180 185 190

Gly Val Ser Gly Ala Ile Gln Ile Asn Gly Pro Ala Ser Leu Pro Tyr
195 200 205

Asp Ile Asp Leu Gly Val Leu Pro Leu Xaa Asp Trp Tyr Tyr Lys Ser
210 215 220

Ala Asp Gln Leu Val Ile Glu Thr Leu Xaa Lys Gly Asn Ala Pro Phe
225 230 235 240

Ser Asp Asn Val Leu Ile Asn Gly Thr Ala Lys His Pro Thr Thr Gly
245 250 255

Glu Gly Glu Tyr Ala Ile Val Lys Leu Thr Pro Asp Lys Arg His Arg
260 265 270

Leu Arg Leu Ile Asn Met Ser Val Glu Asn His Phe Gln Val Ser Leu
275 280 285

Ala Lys His Thr Met Thr Val Ile Ala Ala Asp Met Val Pro Val Asn
290 295 300

Ala Met Thr Val Asp Ser Leu Phe Met Ala Val Gly Gln Arg Tyr Asp
305 310 315 320

Val Thr Ile Asp Ala Ser Gln Ala Val Gly Asn Tyr Trp Phe Asn Ile
325 330 335

Thr Phe Gly Gly Gln Gln Lys Cys Gly Phe Ser His Asn Pro Ala Pro
340 345 350

Ala Ala Ile Phe Arg Tyr Glu Gly Ala Pro Asp Ala Leu Pro Thr Asp
355 360 365

Pro Gly Ala Ala Pro Lys Asp His Gln Cys Leu Asp Thr Leu Asp Leu
370 375 380

Ser Pro Val Val Gln Lys Asn Val Pro Val Asp Gly Phe Val Lys Glu
385 390 395 400

Pro Gly Asn Thr Leu Pro Val Thr Leu His Val Asp Gln Ala Ala Ala
405 410 415

Pro His Val Phe Thr Trp Lys Ile Asn Gly Ser Ala Ala Asp Val Asp
420 425 430

Trp Asp Arg Pro Val Leu Glu Tyr Val Met Asn Asn Asp Leu Ser Ser
435 440 445

Ile Pro Val Lys Asn Asn Ile Val Arg Val Asp Gly Val Asn Glu Trp
450 455 460

Thr Tyr Trp Leu Val Glu Asn Asp Pro Glu Gly Arg Leu Ser Leu Pro
465 470 475 480

His Pro Met His Leu His Gly His Asp Phe Phe Val Leu Gly Arg Ser
485 490 495

Pro Asp Val Ser Pro Asp Ser Glu Thr Arg Phe Val Phe Asp Pro Ala
500 505 510

Val Asp Leu Pro Arg Leu Arg Gly His Asn Pro Val Arg Arg Asp Val
515 520 525

Thr Met Leu Pro Ala Arg Gly Trp Leu Leu Leu Ala Phe Arg Thr Asp
530 535 540

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Asn Pro Gly Ala Trp Leu Phe His Cys His Ile Ala Xaa His Val Ser
545                 550                 555                 560

Gly Gly Leu Ser Val Asp Phe Leu Glu Arg Pro Asp Glu Leu Arg Gly
                565                 570                 575

Gln Leu Thr Gly Glu Ser Lys Ala Glu Leu Glu Arg Val Cys Arg Glu
            580                 585                 590

Trp Lys Asp Trp Glu Ala Lys Ser Pro His Gly Lys Ile Asp Ser Gly
        595                 600                 605

Leu Lys Gln Arg Arg Trp Asp Ala
    610                 615
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CLAIMS

1. A variant of a parent laccase, which variant has laccase activity, and increased oxidation potential and comprises a mutation in a position corresponding to at least one of the following positions:
G511A,V,P,L,I,F,Y,W;
T428A,V,P,L,I,F,Y,W;
S510A,V,P,L,I,F,Y,W;
D106A,V,P,L,I,F,Y,W;
N109A,V,P,L,I,F,Y,W,Q;
L500I,F,Y,W;
A108V,P,L,I,F,Y,W;
G514A,V,P,L,I,F,Y,W;
wherein the parent laccase has the amino acid sequence given in SEQ ID No. 1 or the parent laccase has an amino acid sequence which is at least 80% homologous to SEQ ID No. 1.

2. A variant of a parent laccase, which variant has laccase activity and an altered pH optimum and comprises a mutation in a position corresponding to at least one of the following positions:
192-193;
234-236;
269;
293-294;
364-365;
372-373;
426-433;
503-513;
wherein the parent laccase has the amino acid sequence given in SEQ ID No. 1 or the parent laccase has an amino acid sequence which is at least 80% homologous to SEQ ID No. 1.

3. A variant of a parent laccase, which variant has laccase activity and an altered mediator efficiency and comprises a mutation in a position corresponding to at least one of the

following positions:
185-194;
235;
293-294;
365-373;
427-429;
505;
507-508;
510-511;
wherein the parent laccase has the amino acid sequence given in SEQ ID No. 1 or the parent laccase has an amino acid sequence which is at least 80% homologous to SEQ ID No. 1.

4. A variant of a parent laccase, which variant has laccase activity and an altered O_2/OH^--pathway and comprises a mutation in a position corresponding to at least one of the following positions:
A506E;
N109D;
H93E;
H95E;
M433E;
M480E;
wherein the parent laccase has the amino acid sequence given in SEQ ID No. 1 or the parent laccase has an amino acid sequence which is at least 80% homologous to SEQ ID No. 1.

5. A variant according to any of claims 1-4, wherein the parent laccase is derived from *Myceliophthora*.

6. A variant according to any of claims 1-4, wherein the parent laccase is derived from *Scytalidium*.

7. A variant according to claim 5, wherein the parent laccase is a *Scytalidium thermophilum* laccase with the sequence ID No. 2.

8. A DNA construct comprising a DNA sequence encoding a laccase variant according to any of claims 1-7.

9. A recombinant expression vector which carries a DNA construct according to claim 8.

10. A cell which is transformed with a DNA construct according to claim 8 or a vector according to claim 9.

11. A cell according to claim 10, which is a microorganism.

12. A cell according to claim 11, which is a bacterium or a fungus.

13. A cell according to claim 12, which is an *Aspergillus niger* or an *Aspergillus oryzae* cell.

14. Use of a laccase variant according to any of claims 1-7 for oxidizing a substrate.

15. Use of a laccase variant according to any of claims 1-7 for dye transfer inhibition.

16. Use of a laccase variant according to any of claims 1-7 for bleaching textiles, in particular for bleaching denim.

17. A detergent additive comprising a laccase variant according to any of claims 1-7 in the form of a non-dusting granulate, a stabilised liquid or a protected enzyme.

18. A detergent additive according to claim 17, which additionally comprises one or more other enzyme such as a protease, a lipase, an amylase, and/or a cellulase.

19. A detergent composition comprising a laccase variant according to any of claims 1-7 and a surfactant.

20. A detergent composition according to claim 19 which additionally comprises one or more other enzymes such as a protease, a lipase, an amylase and/or a cellulase.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00067

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/02
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9709431 A1 (NOVO NORDISK BIOTECH, INC.), 13 March 1997 (13.03.97), see claims 20, 26-30 --	2,5
Y	WO 9533836 A1 (NOVO NORDISK BIOTECH, INC.), 14 December 1995 (14.12.95) --	1-5,8-20
Y	WO 9533837 A1 (NOVO NORDISK BIOTECH, INC.), 14 December 1995 (14.12.95) --	1-4,6-20
Y	WO 9511296 A1 (DEGUSSA AKTIENGESELLSCHAFT), 27 April 1995 (27.04.95), see abstract --	1-20

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 May 1998	17 -06- 1998
Name and mailing address of the ISA/	Authorized officer

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00067

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal of Biological Chemistry, Volume 263, No 2, January 1988, Ursula A. Germann et al, "Characterization of Two Allelic Forms of Neurospora crassa Laccase. Amino- and carboxyl-terminal processing of a precursor" page 885 - page 896 --	1-20
Y	The Journal of Biological Chemistry, Volume 265, No 25, Sept 1990, Yasushi Kojima et al, "Cloning, Sequence Analysis, and Expression of Ligninolytic Phenoloxidase Genes of the White-rot Basidiomycete Coriolus hirsutus", page 15224 - page 15230 --	1-20
A	Biochimica et Biophysica Acta, Volume 1292, 1996, Feng Xu et al, "A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability", page 303 - page 311, see abstract --	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00067

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark n Protest ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Ir. ational application No.
PCT/DK 98/00067

According to rule 13.2, an international application shall relate to one invention only or a group of inventions linked by one or more of the same or corresponding "special technical features" i.e. features that define a contribution which each of the inventions makes over prior art.

A search for this "special technical feature" mentioned in PCT Rule 13.2 among the independent claims did not reveal such a unifying technical feature.

According the following inventions were found:

1) Claim 1 and part of claims 5-20 directed to a laccase variant havin an increased oxidation potential

2) Claim 2 and part of claims 5-20 directed to a laccase variant having an altered pH optimum

3) Claim 3 and part of claims 5-20 directed to a laccase variant having an altered mediater efficiency

4) Claim 4 and part of claims 5-20 directed to a laccase variant having an altered O_2/OH-pathway.

INTERNATIONAL SEARCH REPORT

Information on patent family members

29/04/98

International application No.
PCT/DK 98/00067

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9709431 A1	13/03/97	AU 7154096 A	27/03/97
WO 9533836 A1	14/12/95	AU 2656595 A	04/01/96
		CA 2191718 A	14/12/95
		EP 0765394 A	02/04/97
		FI 964808 A	02/12/96
		JP 10501137 T	03/02/98
WO 9533837 A1	14/12/95	AU 2656695 A	04/01/96
		EP 0763115 A	19/03/97
WO 9511296 A1	27/04/95	AU 7938294 A	08/05/95
		EP 0724629 A	07/08/96
		JP 9504426 T	06/05/97